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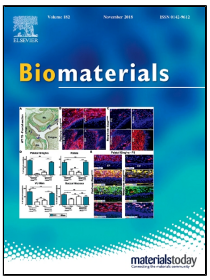

Towards biobanking technologies for natural and bioengineered multicellular placental constructs

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Towards biobanking technologies for natural and bioengineered multicellular placental constructs

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Abstract

Clinical application of a large variety of biomaterials is limited by the imperfections in storage technology. Perspective approaches utilizing low-temperature storage are especially challenging for multicellular structures, such as tissues, organs, and bioengineered constructs. Placenta, as a temporary organ, is a widely available unique biological material, being among the most promising sources of various cells and tissues for clinical and experimental use in regenerative medicine and tissue engineering. The aim of this study was to analyze the mechanisms of cryoinjuries in different placental tissues and bioengineered constructs as well as to support the viability after low temperature storage, which would contribute to development of efficient biobanking technologies. This study shows that specificity of cryodamage depends on the structure of the studied object, intercellular bonds, as well as interaction of its components with cryoprotective agents. Remarkably, it was possible to efficiently isolate cells after thawing from all of the studied tissues. While the outcome was lower in comparison to the native non-frozen samples,

the phenotype and expression levels of pluripotency genes remained unaffected. Further progress in eliminating of recrystallization processes during thawing would significantly improve biobanking technologies for multicellular constructs and tissues.

Keywords

Placental tissues; amniotic membrane; alginate microspheres; bioengineered constructs; multipotent stromal cells; biobanking

Introduction

Significant amounts of biomaterial cannot be used for clinical and research purposes simply due to the shortcomings in storage technologies. A promising solution to this problem is the application of approaches utilizing low temperatures. Developed methods allow successful preservation of cell suspensions and some tissues, but very few works are related to multicellular bioengineered constructs [1-3].

Placenta, foetal membranes, umbilical cord, hematopoietic and multipotent stromal cells (MSCs) of placental origin are increasingly attracting the attention of researchers and clinicians worldwide [4-7]. Components of placental complex are readily applied in medicine as well as undergo research and pre-clinical trials in regenerative approaches and tissue engineering [7].

In particular, amniotic membrane has been successfully used in clinical practice in surgery as a wound dressing for over a century [7, 8]. Currently, the most frequent application with expressed long-term clinical effects is seen in ophthalmology. These effects are determined by a row of unique properties of the amniotic membrane, including the tissue transparency, the ability to suppress corneal vascularization, as well as the ability to stimulate proliferation and migration of limbal stem cells [9, 10]. In addition to ophthalmology, amniotic and chorionic membranes showed pronounced therapeutic outcome

in the treatment of enterocutaneous fistula, non-healing trophic ulcers, prevention of adhesions, orthopedic disorders, and etc. [7, 11, 12]. Here, therapeutic effects of placental membranes are warranted among others by the action as a biological dressing, antiseptic properties along with suppression of inflammation and scarring, as well as the capability to activate epithelialization [7, 13, 14]. Placental tissue fragments have been successfully applied in experimental studies for the treatment of variety of chronic inflammatory diseases, as well as for the correction of male infertility [7, 15]. Umbilical cord is highly regarded as a source of large amounts of hematopoietic stem cells and MSCs which, in turn, are potentially considered as an alternative to transplantation of bone marrow cells [16, 17].

Among numerous advantages of placental material are the possibility of obtaining large amounts of tissues, including autologous, without the invasive or traumatic procedures; high proliferation potential and low immunogenicity of the cells of placental origin [18, 19]; as well as the capacity to isolation of MSCs in amounts sufficient for therapeutic purposes [20]. Advanced studies have been performed with cellular suspensions isolated from placenta, umbilical cord, and foetal membranes [21, 22]. Such cells have been successfully phenotyped; their properties and perspectives of applicability in regenerative medicine are described; a range of efficient cryopreservation protocols has been developed [23-25]. Great therapeutic potential of MSCs was also recognized for the use in generation of artificial multicellular bioengineered constructs [26]. Successful viable tissue engineered heart valves and cardiovascular implants were generated in experimental studies [27, 28]. Novel technologies are being developed for bioengineered alginate carriers containing viable MSCs of placental origin in order to address potential immunological issues and stable quality outcome [2, 3, 26, 29].

At the same time there is a high demand for development of efficient technologies for cryopreservation and storage of placental tissue, umbilical cord, amniotic membrane, and bioengineered multicellular constructs based on

the cells of placental origin [2, 30-32]. Firstly, this may allow direct cryoconservation at the maternity hospitals, when, e.g. there are obstacles to immediate delivery of the material to a laboratory for the further processing. This could be especially important for placental tissue intended for further isolation of viable stem cells. Secondly, this will open wide horizons to the highly promising application of amniotic membranes for therapeutic purposes. Thirdly, establishment of low-temperature banks and cryopreservation expertise would be required for generation and application of multicellular bioengineered constructs. At fourth, relevant programs for cryopreservation of placental components are required for allowing the necessary time to conducting the novel diagnostic approaches in clinics. Furthermore, comparison of cryoinjury mechanisms of various tissues would lead to development and optimization of cryopreservation protocols for various tissue types, and allow creating bioengineered constructs with cryo-stable properties.

Placental tissues are highly relevant as a model for studying the mechanisms of cryoinjuries with potential for successful cryopreservation outcome. This is particularly determined by the thinness of the villi and membranes, transparency and loose intracellular substance that allow easy penetration of cryoprotective agents to the cells, as well as possibility for application of microscopic equipment without the necessity for additional tissue fragmentation and processing.

To date, several methods for tissue cryopreservation, including placental tissue, umbilical cord, and placental membranes, have been implemented [18, 25, 33-38]. At the same time, certain critical issues still remain unresolved [1, 36, 39].

Firstly, the mechanisms of cryoresistance and cryoinjury of tissues and bioengineered constructs partially remain unclear [39]. Secondly, there is a virtual absence of comparative studies and standardization of methods and approaches for evaluation of viability and survival of such material after cryopreservation. Thirdly, there is no available data on comparison of

quantitative and qualitative characteristics of cell populations isolated from various types of native (fresh, non-frozen) and cryopreserved tissues. At fourth, efficient and, at the same time, uncomplicated technology for tissues cryopreservation, which would be possible under the conditions of a maternity hospital with the use of existing equipment and under the GMP standards, has not been developed yet [36, 40].

In our previous work, we have studied the viability of placental cells, tissues, and bioengineered constructs before and after cryopreservation [3, 18, 23, 24, 29]. The outcome of cryopreservation of analysed biological objects was shown with different methodological approaches. However, performed studies did not reveal the mechanisms of cryoinjuries and cryoresistance. Therefore, the aim of this work was to study the mechanisms of cryoinjuries and cryoresistance of placental tissues and bioengineered constructs, as well as to obtain viable outcome after low temperature storage.

Materials and methods

Experimental design

Placental villi composed of the loose connective tissue and a large number of cells, *amniotic membrane* with a dense layer of connective tissue and epithelial cells on the surface, and *umbilical cord* tissue, rich of hyaluronic acid, were selected as natural biological objects for analysis of the mechanisms of cryoinjuries and cryoresistance. *Spheroids*, where integrity is ensured by the intercellular interaction, as well as *cells encapsulated into alginate microspheres*, where integrity is ensured by polymerized gel with the virtual absence of cell-cell interactions, were selected as bioengineered constructs. All objects were frozen with application of conventional method for cryopreservation of adherent cell cultures [24]. Cryoinjuries of the studied objects were analysed with cryomicroscopy. Viability was evaluated with confocal microscopy by the methods of vital staining with fluorescein diacetate (FDA) and ethidium bromide (EB). Besides, cell populations were isolated from

the native as well as cryopreserved material with the following characterization. Furthermore, obtained data was analyzed and correlated with the previously published results of the studies conducted with additional methods [18, 24].

Derivation of placental tissues, cells, and bioengineered multicellular constructs

Human placentas were received after routine Caesarian section from the 18-35 year old women. Material was donated in an anonymized manner with a written informed consent of the patients at the Department of Gynaecology and Obstetrics at Hannover Medical School, Germany (approved by Ethical Commission of Hannover Medical School, Ethic votum No.2396-2014) and in Kharkiv Municipal Maternity Hospital No.1, Ukraine (approved by Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Ethic votum No.2-0306-2013) as donation. The afterbirth was delivered to the laboratory within 1-3 hours in a moist chamber.

For obtaining placental explants, maternal side of placental tissue was dissected into small fragments, up to 10 mm^3 in size, and washed three times in Dulbecco's modified Eagle's medium (DMEM, Lonza, Switzerland). Foetal membranes were washed twice in DMEM; amniotic membrane was separated from chorion and dissected into fragments 25 mm^2 in size. Umbilical cord was cleared from the vessels, dissected into small fragments, up to 3-4 mm^3 in size, and washed twice in DMEM.

Primary cell cultures from native and frozen placental tissues and amniotic membranes were derived with application of enzymatic method, as described before [23]. In brief, placental tissue and amniotic membranes were washed in phosphate-buffered saline (PBS) supplemented with 10% Ciprofloxacin (Fresenius Kabi, Germany) followed by mechanical disintegration and incubation in 0.25% trypsin for 1 hour at 37°C. After trypsin digestion the cells were filtered through the 100 μm cell strainer (BD Biosciences, USA) and

obtained cell suspension was spun down at 300 x g for 10 min (Heraeus Multifuge 1S-R, Thermo Fisher Scientific, USA), resuspended in culture medium with the following cell culturing (described below).

Primary culture from umbilical cord tissue was obtained by explant method [38]. In brief, umbilical cord tissue was washed with PBS and dissected into 3-4 mm^3 size fragments. Explants were cultivated up to 3 weeks with medium replacement every 3-4 days. Tissue pieces were removed from the culture dish one week after the cell outgrowth was observed.

All obtained cell types were passaged with trypsin and analysed at passages 3 and 4. Cell populations acquired from placenta, amnion, and umbilical cord were characterized and phenotyped for their MSC origin as described below in the corresponding section.

Spheroids were obtained with a hanging drop method [41]. In brief, placental cells at concentration of 5×10^5 cells/ml were applied within 35 µl drops on the surface of 60 mm tissue culture dish (CELLSTAR®, Greiner BioOne, Austria), inverted upside down for and incubated at 37°C with 5% CO_2 and 95% humidity for 24 hours. After that, formed spheroids were harvested for the further experimental studies.

Alginate microspheres were obtained with encapsulation method [2]. In brief, suspension of placental cells was spun down at 300 x g for 5 min and the cell pellet was resuspended in concentration of 1×10^6 cells/ml in 1% alginate dissolved in 0.9% NaCl. Suspension was placed in a sterile syringe and drop sprayed into 100 mM $CaCl_2$ cross-linking solution, followed by polymerization of alginate and formation of microspheres 500-750 µm in diameter containing encapsulated cells. Obtained microspheres were transferred to the cell culture medium for the further experimental studies.

Cell and tissue culture

Cells, tissue fragments, as well as multicellular bioengineered constructs were cultured under sterile conditions in humidified CO_2 incubator (Thermo

Fisher Scientific, USA) at 5% CO_2 and 37ºC in DMEM, supplemented with 1% (v/v) 100 mM sodium pyruvate solution (Lonza, Switzerland), 10% (v/v) fetal bovine serum (FBS, Lonza, Switzerland), 1% (v/v) antibiotic-antimycotic solution (BioWest, France), and 1% (v/v) ascorbic acid (Sigma-Aldrich, USA) in 10 cm tissue culture plates (CELLSTAR®, Greiner BioOne, Austria). 0.25% Trypsin-EDTA solution was applied for harvesting and passaging of the cells.

Cryopreservation

CPA medium was composed of DMEM with high glucose content, L-glutamine and sodium pyruvate, supplemented with 10% DMSO (Sigma-Aldrich, USA) and 10% FBS. The samples were exposed in cryopreservation medium for 10 min at 4°C and placed into 1.8 ml cryovials (Nunc™, Thermo Fisher Scientific, USA) with subsequent cooling with the rate of 1°C/min down to -80°C in the programmable freezer "ЗП-10" (BioCold Special Designing and Technical Bureau, Ukraine), or in isopropyl alcohol based freezing container (Nalgene® Mr. Frosty™, Thermo Fischer Scientific, USA) followed by immersion to the liquid nitrogen. The samples were rapidly thawed at +37°C in a water bath with subsequent washing with a culture medium for removing DMSO by centrifugation for 5 min at 300 x g in the case of cells or sedimentation for 5 min in the case of tissues and bioengineered constructs. Native non-cryopreserved samples were used as a positive control; samples, directly immersed in the liquid nitrogen without cryoprotective agents, were used as a negative control.

Phenotyping of cells derived from native and cryopreserved placental tissues

Phenotypic characterization of the cells derived from the native and cryopreserved placental tissues was performed by flow cytometry analysis for the specific cell surface markers: CD29 (integrin beta-1, ITGB1), CD34

(hematopoietic progenitor cell antigen CD34), CD44 (homing cell adhesion molecule, HCAM), CD73 (ecto-5'-nucleotidase, NT5E), CD105 (endoglin, ENG), CD106 (vascular cell adhesion molecule 1, VCAM), and CD166 (activated leukocyte cell adhesion molecule, ALCAM). The cells were aliquoted equally into fluorescence-activated cell sorting (FACS) tubes (Corning, USA) and stained with corresponding antibodies according to the standard protocol. In brief, the cells were resuspended in 100μL PBS, then stained with phycoerythrin (PE)-conjugated anti-CD29 (clone MAR4; BD Biosciences, USA), allophycocyanin (APC)-conjugated anti-CD34 (clone HPCA2; BD Biosciences, USA), PE-conjugated anti-CD44 (clone 515; BD Biosciences, USA), PE-conjugated anti-CD73 (clone AD2; BD Biosciences, USA), PE-conjugated anti-CD105 (clone 43A3; BioLegend, USA), PE-conjugated anti-CD106 (clone 51-10C9; BD Biosciences, USA), and PE-conjugated anti-CD73 (clone 3A6; BD Biosciences, USA) antibodies individually for each marker and incubated for 15 min at room temperature in the absence of light. After incubation the cells were washed with 1ml PBS and analyzed with a FACSCanto™ flow cytometry system (Becton Dickinson, USA) with a rate of 10,000 events per measurement. Similar staining procedures have been performed with APC-conjugated Mouse IgG1 isotype control (clone X40; BD Biosciences, USA) and PE-conjugated Mouse IgG1 isotype control (clone MOPC-21; BD Biosciences, USA) to differentiate non-specific background signal from the specific antibody signal. Non-stained cells were used as a negative control to eliminate the possibility of autofluorescence. Forward scatter (FSC) and side scatter (SSC) profiles were used in gating to distinguish cells with the size appropriate to MSCs. Percentage of cells positive for each marker was calculated from the cells gated according to the expected FSC and SSC parameters (P1 gate).

Evaluation of viability

Viability of cells in the samples was evaluated by confocal microscopy with combined staining with fluorescein diacetate and ethidium bromide dyes by the previously described method [2]. In brief, FDA was dissolved in acetone in concentration of 5 mg/ml and EB was dissolved in ethanol in concentration of 3 mg/ml. A working solution was composed of 3 ml PBS, 5 µl FDA, and 5 µl EB. Studied samples were incubated in the working solution for 10 minutes prior to microscopy. Images were obtained with inverted laser scanning confocal microscope Zeiss LSM 510 META (Carl Zeiss, Germany) and analyzed with LSM 510 ver.4.2 software (Carl Zeiss, Germany). The images were obtained at 488 nm excitation wavelength, with absorption at 508-550 nm for FDA and 593-646 nm for EB. The cells stained with FDA were considered as viable and stained with EB as dead. Laser aperture (pinhole) was 150 nm. Individual sections of three-dimensional projections were analyzed.

Metabolic activity

MTT Test. Metabolic activity of the samples was measured with application of MTT test “CellTiter 96® Non-Radioactive Cell Proliferation Assay” (Promega, USA) according to the manufacturer’s protocol, considering peculiarities of the studied samples. In particular, 15 mg of the studied tissue was placed per well of the 24-well plate (CELLSTAR®, Greiner BioOne, Austria) in 500 µl of the culture medium with the following addition of 75 µl of MTT reagent and incubation for 4 hours at 37°C in humidified CO_2 incubator. Afterwards, the medium was removed and formazan was extracted from the tissue by adding 1 ml of 96% ethanol for 30 minutes at 37 °C. Formazan concentration was assessed by measuring optical density (OD) of the supernatant with PV 1251C spectrophotometer (Solar, Belarus) at a wavelength of 570 nm. Spheroids with a total input of 1 x 10^6 cells per well were placed on a 96-well plate (CELLSTAR®, Greiner BioOne, Austria) in 100 µl culture medium per well. 15 µl of MTT reagent per well was added with the following incubation for 4 hours at 37°C in humidified CO_2 incubator. Then 100

µl of Stop Mix reagent per well was added and incubated further for 1 hour at room temperature in the absence of light. Afterwards, formazan concentration was measured with Bio-Rad 680 XR Microplate Reader (Bio-Rad, USA) at 570 nm wave length.

Resazurin reduction test. Additionally, resazurin reduction test was performed. 15 mg of the studied tissue in 500 µl of the culture medium was placed per well of the 24 well plate with the following addition of 200 µL resazurin solution (Sinbias, Russia) in PBS at 0.15 mg/ml concentration and incubation for 24 hours at 37°C in humidified CO_2 incubator. Afterwards, OD was measured spectrophotometrically at a wavelength of 570 nm. Spheroids and alginate microspheres were placed on a 96-well plate in 100 µl culture medium per well. 50 µl of resazurin solution was added per well with the following incubation for 24 hours at 37°C in humidified CO_2 incubator. Incubation was followed by measuring of the OD at a wavelength of 570 nm with a plate reader.

Cryomicroscopy

Cryomicroscopic analysis was performed in cryomicroscopic chamber complex KP 6 (SDTB IPC&C, Ukraine) based on MBI 15U 4.2 microscope (LOMO, Russia). The images were recorded with UCMOS 3100 digital camera (SIGETA, China) with Toup View v.3.7 software (Hangzhou ToupTek Photonics Co. Ltd, China). 25 µl of the medium containing studied sample was placed in the cryomicroscopic chamber and cooled with the rate of 1 °C/min down to -100°C.

In order to achieve transparency in the samples with alginate encapsulated cells, 25 µl of 1% sodium alginate in 0.9% NaCl with cells at concentration of 10^6 cells/ml were placed in the cryomicroscopic chamber and polymerized with 10% $CaCl_2$ in 0.9% NaCl solution. PBS was not used for polymerization as phosphates modify the alginate's transparency. Afterwards, the resulting film was topped with cryopreservation medium containing 10%

DMSO and covered with a coverslip. Additionally, cryomicroscopy without DMSO was carried out.

In order to determine the effect of DMSO on the alginate solution, 1% solution of sodium alginate was added dropwise to DMSO in concentrations of 50, 25, 20, 15, and 10% in 0.9% NaCl. Additionally, 1% solution of sodium alginate was added dropwise to DMSO solutions in concentrations of 50, 25, 20, 15, 10% in EDTA.

The images were analyzed with application of ImageJ 1.6.024 Software (NIH, Public domain).

Isolation of RNA and cDNA synthesis

Total RNA was purified from the studied samples with application of QIAGEN RNeasy RNA Isolation Kit (QIAGEN, Germany) according to the manufacturer's protocol. Briefly, 2 x 10^6 cells were resuspended in RLT buffer with 1% β-mercaptoethanol and 70% ethanol was added to the cellular lysate to condition selective binding of RNA to the RNeasy membrane. Then the samples were applied to the RNeasy Mini spin column, where RNA is binding to the membrane followed by washing away of contaminants and final elution of high-purity RNA with RNase-free dH_2O. All bind, wash, and elution steps were performed by centrifugation at 8000 x g in a microcentrifuge. Concentration of the RNA was measured with NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) was used to transcribe the extracted RNA into cDNA. Adding of Oligo(dT) primers (Thermo Fisher Scientific, USA) ensured that only the mRNA was transcribed.

Quantitative PCR

Quantitative polymerase chain reaction (qPCR) was established as described before [42]. In brief, 1 µl of the sample containing 10 ng cDNA was

added to 19 µl SYBR Green master mix (Life Technologies, USA) supplemented with corresponding forward and reverse primers. Concentration of each primer pair was optimized by titration and analysis of the melting curve for specificity and efficiency. The quantitative PCR was performed with application of StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA). Cycling conditions included 10 minutes of precycling at 95°C and 40 cycles of denaturation at 95°C for 15 seconds with annealing at 60°C for 1 minute. Obtained melting curves were analyzed for specificity of the products. Ribosomal housekeeper gene RPS29 showed stability within the range of studied cells and tissues and was selected for ΔCt normalization with bone marrow MSCs control for $2^{-\Delta\Delta Ct}$ analysis. Design of oligonucleotides was performed on the basis of human consensus sequences with application of the National Center for Biotechnology Information (NCBI) and Wellcome Trust Sanger Institute/The European Bioinformatics Institute (Ensembl) databases. In order to exclude gDNA contamination, the sequences from two different exons were used where possible.

Data analysis and statistics

All data was collected from 3 to 5 independent experiments in triplicates ($n \geq 3$). Statistically significant conclusions were obtained with analysis of mean values and standard deviation calculations (mean ± SD) with Mann-Whitney test and Fisher's method. Parametric changes were considered statistically significant at $p<0.05$. Past V. 3.15 Software (UiO, Norway) and GraphPad Prism 5.02 (GraphPad Software Inc., USA) were used for statistical calculations and data analysis.

Results

Cryopreservation of placenta tissue

Cryomicroscopic studies of crystallization processes in the placenta villi showed no visible alterations until lowering the temperature down to -14°C. At this temperature point sharp outbreak of the crystal formation was observed, which was expressed in darkening of the tissue and formation of small crystals around the tissue (Figure 1). The effect of darkening and reduced transparency is caused by the drastic increase in the number reflecting surface, indicating the large number of crystals in the sample, which complicate cryomicroscopic analysis. Visually noticeable crystallization processes continue down to -30°C - -40°C. The crystals outside of the tissue significantly increase in size to 12.4 ± 1.9 μm, while visible crystals 2.3 ± 0.5 μm in size start to appear inside the tissue. Further lowering of the temperature down to -100°C did not result in visible changes. During thawing, the recrystallization effect in the tissue was observed at elevation of the temperatures from -40°C to -20°C. The size of the crystals associated with tissue in this temperature range was 4.4 ± 1.5 μm. Melting of the ice crystals started with the increase of temperature above -20°C and finished at -7°C - -6°C. The structure as well as the surface size of non-stained samples did not vary significantly before and after cryopreservation.

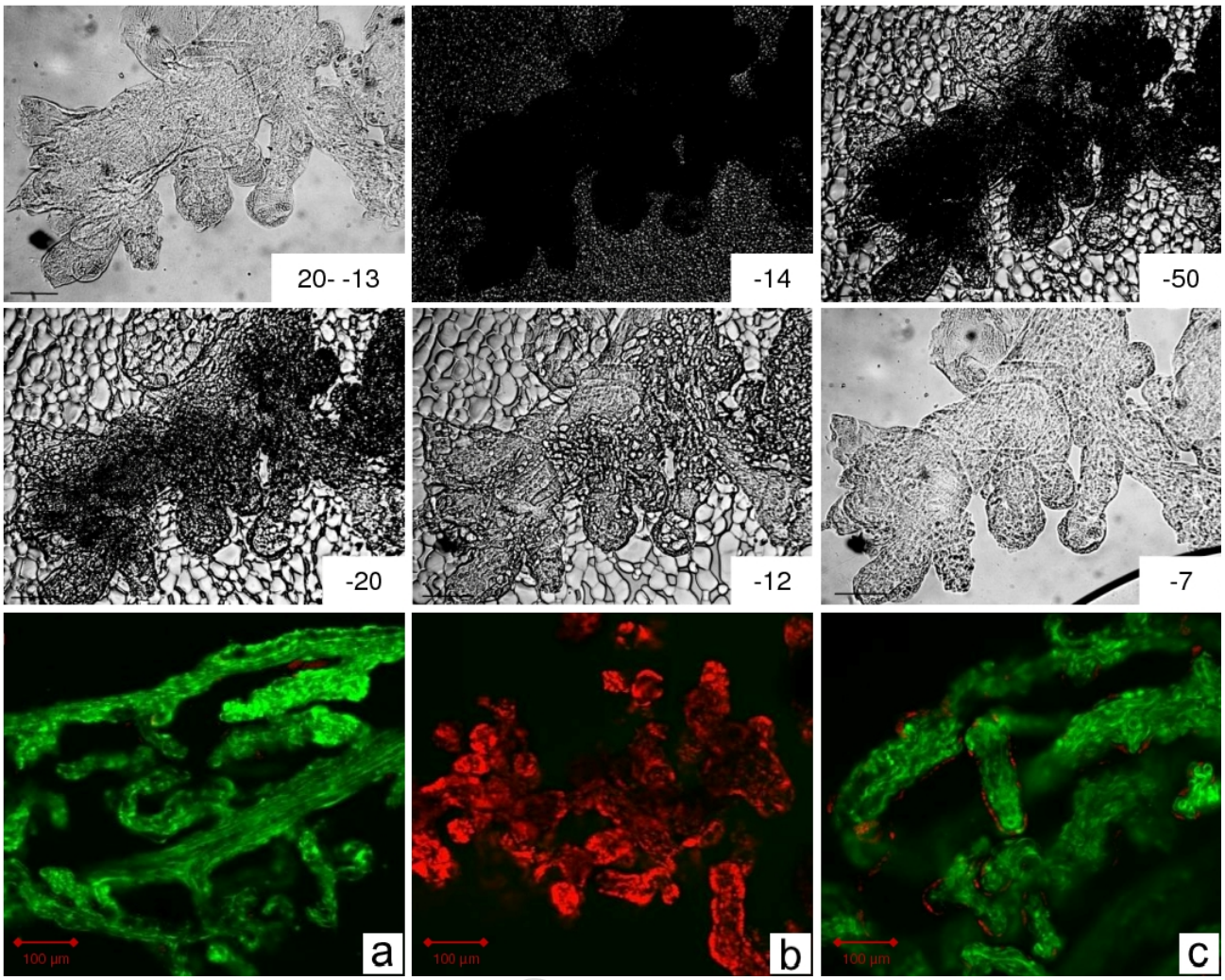

Figure 1. **Morphological study of placental villi tissue before, after, and during cryopreservation procedures.** Cryomicroscopic analysis: freezing-thawing of placental villi tissue (numbers indicate the temperature, °C), scale bars – 50 µm; confocal microscopy: a - native tissue, b - negative control, c - after cryopreservation. Green colour fluorescence (FDA) represents viable cells, red colour fluorescence (EB) represents dead cells.

Confocal microscopy of the native sample revealed staining of majority of the cells with FDA dye, whereas EB have stained less than 1% of the cells (Figure 1). At the same time, 100% of the cells were stained with EB in the negative control (Figure 1b). Majority of the cells in the samples after cryopreservation were stained with FDA, with EB staining only several solitary cells (Figure 1c). However, it was observed that EB poorly penetrates inside the villi, while FDA poorly stains the syncytium layer.

Metabolic activity of placental villi tissue after cryopreservation did not differ significantly to metabolic activity of the native samples when analysed with MTT test (Table 1). At the same time, resazurin reduction test showed slight decrease of metabolic activity after cryopreservation (Table 1).

Table 1. **Metabolic activity of various placental tissues and multicellular bioengineered constructs before and after cryopreservation procedures.**

Sample name	Contidion	MTT, AU	Resazurin, AU
Placental villi	native	3252.7±130.8*	59.7±4.0*
	after cryopreservation	3193.2±214.3*	39.3±5.0* **
	negative control	197.6±10.9	4.9±0.3
Amniotic membrane	native	984.6±38.8*	44.8±33.01*
	after cryopreservation	517.6±33.2* **	21.04±2.6* **
	negative control	197.6±10.9	4.9±0.3
Umbilical cord	native	484.±20.4*	27.9±2.3*
	after cryopreservation	504.8±20.7**	9.3±2.09* **
	negative control	197.6±10.9	4.9±0.3
Spheroids	native	0.8±0.03*	33.0±3.1*
	after cryopreservation	0.72±0.03* **	46.3±3.7* **
	negative control	0.35±0.03	4.9±0.3
Alginate microspheres	native	-	17.4±1.0*
	after cryopreservation	-	11.9±1.1* **
	negative control	-	4.9±0.3

* – significant difference to negative control, p <0,05; ** – significant difference to native (non-cryopreserved) control, p <0,05.

Cryopreservation of amniotic membrane

Cryomicroscopic studies of amniotic membranes revealed abrupt darkening in the field of view at -14°C with detection of single crystals in the tissue. Further lowering of the temperature did not result in visible changes. Warming of the sample resulted in recrystallization effects in the tissue, which was expressed in lightening and visualisation of separate crystals located in the various levels of focal points of the microscope (Figure 2). At -20°C the crystals were maximally transparent and had the size of 13.5 ± 1.5 μm in length and 5.9 ± 2.1 μm in width. These crystals were oriented along the fibres and started to melt. The melting process was completed at -5°C - -6°C. No obvious morphological differences between cryopreserved and non-cryopreserved non-stained samples were observed.

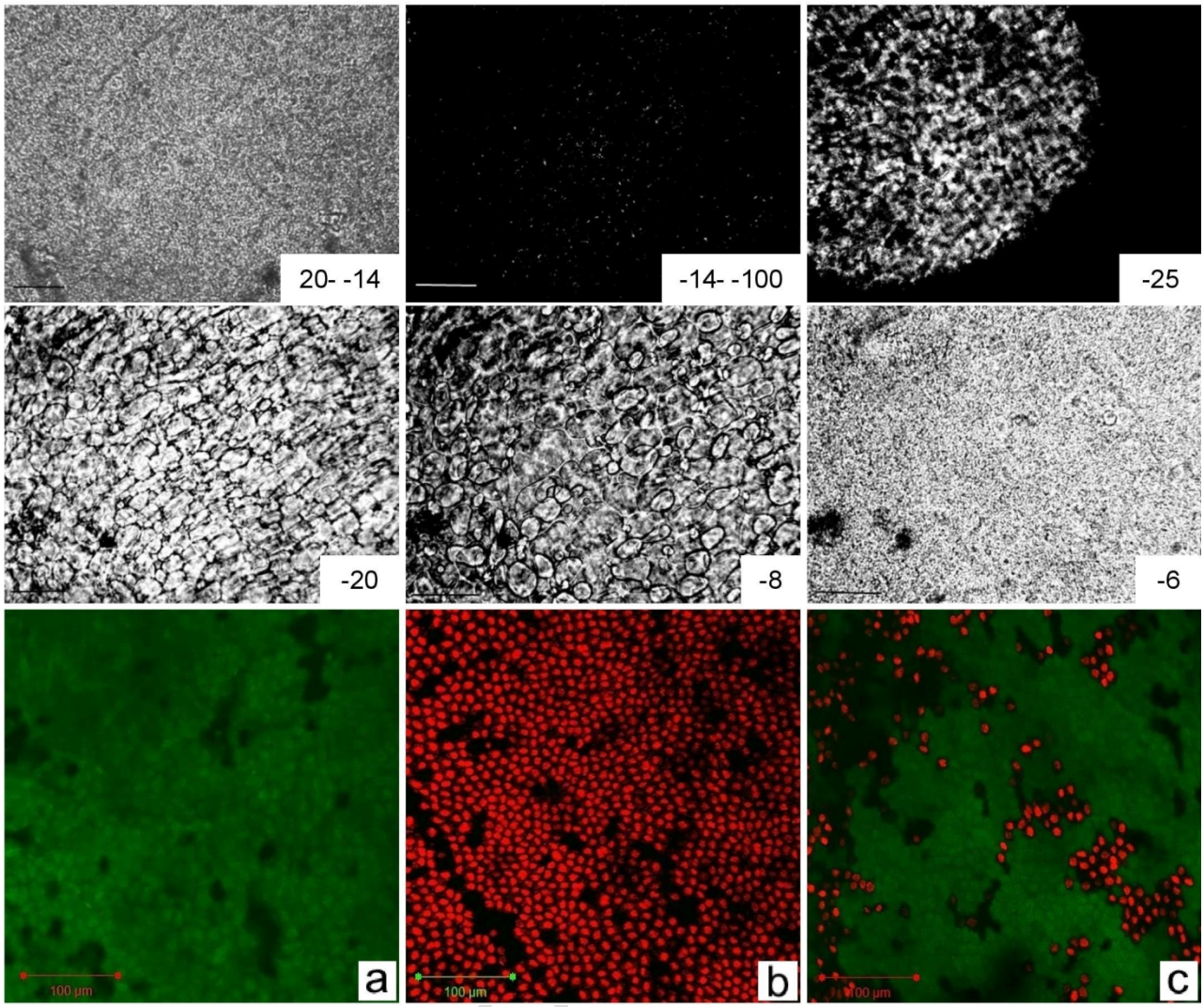

Figure 2. **Morphological study of amniotic membrane tissue before, after, and during cryopreservation procedures.** Cryomicroscopic analysis: freezing-thawing of foetal membrane tissue (numbers indicate the temperature, °C), scale bars – 50 µm; confocal microscopy: a - native tissue, b - negative control, c - after cryopreservation. Green colour fluorescence (FDA) represents viable cells, red colour fluorescence (EB) represents dead cells.

Confocal microscopy studies were carried out on the surface layer of amniotic membrane, where the vast majority of cells are located. In the lower layers of amniotic membrane the connective tissue is prevailing and the number of cells is minor, leading to insufficiency of the staining. In the native sample majority of the cells were stained with FDA. A small amount of non-stained cells was regarded as being dead or lost during preparation of the

sample due to mechanical damage. In certain areas of some samples desquamation of the epithelium was observed (Figure 2a). In the negative control all cells were stained with EB, except the desquamated area (Figure 2b). In the samples after cryopreservation majority of the cells were stained with FDA. EB stained 18.6 ± 3.6% of the cells, varying in the different fields of view from 39 to 10% (Figure 2c).

Metabolic activity of amniotic membrane significantly dropped after cryopreservation in comparison to the native samples according to both MTT and resazurin reduction tests (Table 1).

Cryopreservation of umbilical cord

Tissue density and structure of umbilical cord did not technically allow receiving the layers thin enough for cryomicroscopic analysis. Confocal microscopy revealed that FDA stains the tissue in a diffusive manner, without penetrating into individual cells (Figure 3a). In our opinion, the difference in staining could be due to the higher hydrophilicity of EB molecules. The number of EB stained cells in the field of view of negative control was considered as 100%. In the native non-cryopreserved samples 52.3 ± 4.7% of cells in the field of view were stained with EB, which may be associated with cell damage during dissection of a very dense umbilical cord tissue. After cryopreservation the number of cells stained with EB reached 76.2 ± 7.3%.

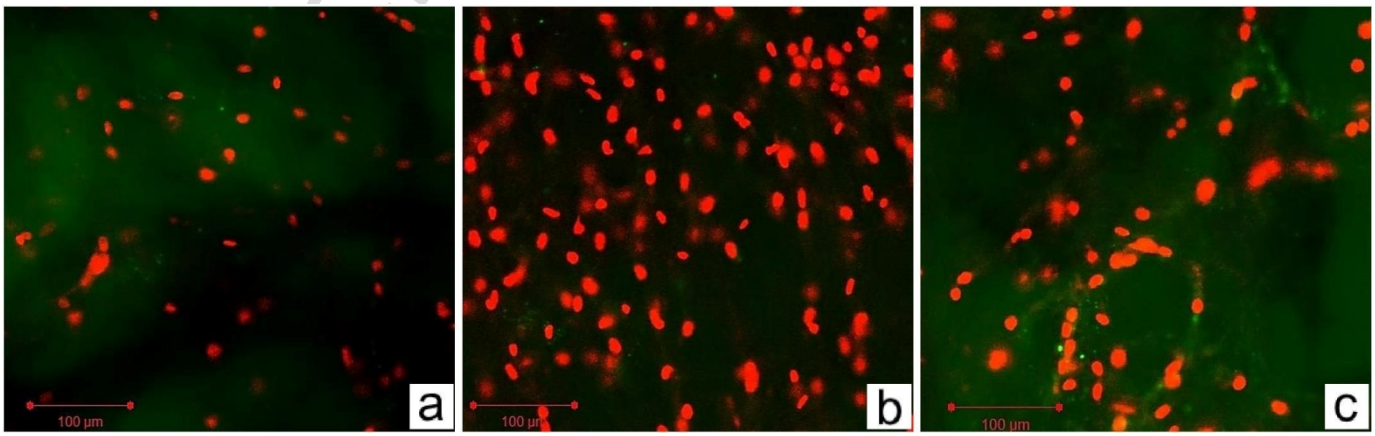

Figure 3. **Morphological study of umbilical cord tissue before and after cryopreservation procedures.** Confocal microscopy of umbilical cord tissue: a - native tissue, b - negative control, c - after cryopreservation. Green colour fluorescence (FDA) represents viable cells, red colour fluorescence (EB) represents dead cells.

Interestingly, umbilical cord tissue showed slight increase of metabolic activity after cryopreservation according to the MTT test, while significant three-fold decrease according to the resazurin reduction (Table 1). The decrease in values of resazurin reduction test alongside the increase in MTT reaction may be explained by necrotic and apoptotic processes in the cells in the first hours after thawing and their progressive loss of mitochondrial activity within one day, since the experimental reaction in the MTT test is performed within 4 hours, while resazurin reduction test that we applied lasts 24 hours [23, 24, 43, 44].

Cryopreservation of spheroids

Input of cells in an amount of 1 × 10^6 and application of hanging drop method allowed receiving approximately 800 spheroids 50-150 μm in diameter (Figure 4). Spheroids had rounded shape and were cultured in non-attachment culture plates. However, in order to analyse viability and functionality of the cells in spheroids, they were placed in adherent culture plates and where able to adhere to plastic and form a monolayer.

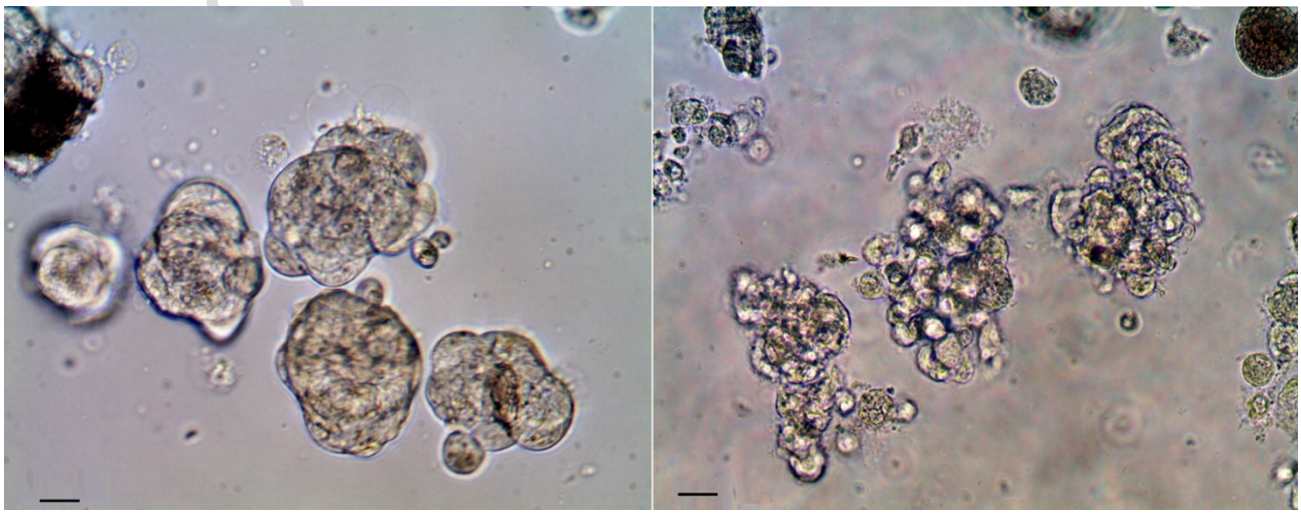

Figure 4. **Morphological study of spheroids with placental cells before and after cryopreservation.** Light microscopy: a – before cryopreservation, b - after cryopreservation. Scale bars 20 μm.

Segregation of separate cells, loss of adhesion, as well as cell death in the outer layers of spheroids was observed after cryopreservation. Additionally, spheroids were disrupted during CPA washing procedures. The total number of spheroids decreased (Table 2). At the same time, metabolic activity did not differ from the control, which may indicate the preservation of individual cells.

Table 2. Total number of spheroids before and after cryopreservation

	Control+	Cryo
Number of spheroids	842.5±65.1	436.4±37.1**

p <0.05; **

Cryomicroscopic analysis during freezing showed initiation of the crystal formation at -14°C, with the crystals inside spheroids and on their surface being smaller in size in comparison to the crystals in the surrounding medium. The processes of crystallization continued with dropping of the temperature down to -40°C, with the crystals associated with spheroids being 5.2 ± 1.5 μm in size, which again was significantly smaller in comparison to the crystals in the surrounding medium. Thawing of the samples was accompanied with crystal melting starting from raising the temperature above -40°C and continued up to -7°C. Pronounced recrystallization processes were observed from -30°C, especially at the surface and inside the spheroids. The maximal crystal size associated with spheroids was observed in the temperature range between -20°C and -15°C. At the beginning of melting processes the average crystal size was 5.2 ± 1.5 μm, later reaching 10.9 ± 4.0 μm. The structure of spheroids was significantly changed after thawing (Figure 5). Deformation, membrane ruptures, and increased granularity of individual cells were observed.

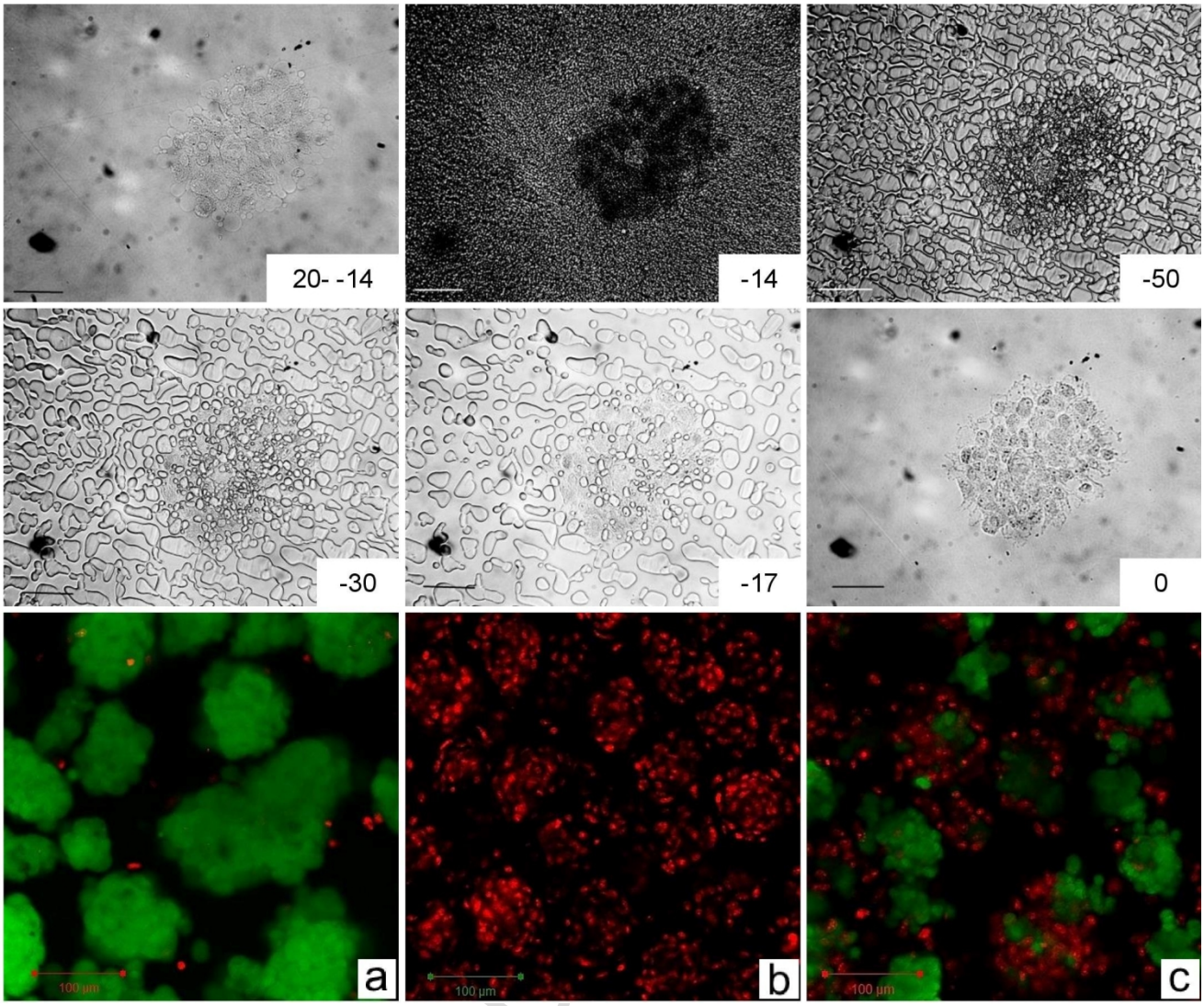

Figure 5. **Morphological study of spheroids with placental cells before, after, and during cryopreservation procedures.** Cryomicroscopic analysis: freezing-thawing of spheroids from placental cells (numbers indicate the temperature, °C), scale bars – 50 μm; confocal microscopy: a - native, b - negative control, c - after cryopreservation. Green colour fluorescence (FDA) represents viable cells, red colour fluorescence (EB) represents dead cells.

Confocal microscopy studies of native spheroids showed staining with FDA and overall integrity of the cells, with only a few single cells stained with EB (Figure 5 a). Complete cell death and complete lack of FDA staining was observed in the negative control (Figure 5, b). After cryopreservation spheroids were partially damaged, resulting in 28.3 ± 3.8% of cells being stained with EB (Figure 5, c).

Metabolic activity of spheroids slightly dropped after cryopreservation according to the MTT test, but was raised up to 30% in comparison to native samples according to resazurin reduction test (Table 1). Values of both tests correspond well to our previous data on placental cell suspensions [24]. Again, certain variances in the outcome of MTT and resazurin reduction tests may be explained by methodological differences connected with incubation period of reagent components (being 4 hours and 24 h, respectively).

Cryopreservation of alginate microspheres

Cryomicroscopic analysis of alginate preparations during freezing revealed the initiation of ice crystal formation at -10°C. Large ice crystals have been formed until the end of crystallization process at -30°C (Figure 6). At this point the whole area of the studied preparation was divided into zones with approximately 75% of the surface area occupied by the large crystals up to 137.9 ± 42.2 μm in size as well as with the small crystals up to 2.6 ± 0.38 μm. The melting process began with the raise of a temperature up to -30°C and already at -20°C the ice completely disappeared in the areas with small crystals. Complete melting occured at -7°C. Comparison of the stained cells before and after cryopreservation revealed that the most damaged cells were previously located on the borders of large crystals. Damaged cells, size change, as well as debris in the alginate cavities were observed. However, the cells located in the central areas did not have visual changes.

Additionally, in order to determine the nature of emergence of two different types of ice crystals in these samples, cryopreservation of the alginate films in cell culture medium without DMSO was performed (Figure 6). In this case, initiation of the ice crystal formation was observed at -17°C, followed by ending at -19°C with development of crystals 45.3 ± 23.2 μm in size. The crystals looked similar on the entire surface. Melting of crystals started at raise of the temperature to -18°C, finishing at -9°C. After thawing the cells either

decreased in size or their remnants were present as debris in the alginate cavities.

Figure 6. **Morphological study of alginate microspheres with placental cells before, after, and during cryopreservation procedures.** Cryomicroscopic analysis: freezing-thawing of alginate microspheres with placental cells (numbers indicate the temperature, °C), scale bars – 50 μm. First two rows from the top - cryopreservation with DMSO, two lower rows - cryopreservation without DMSO. Confocal microscopy: a - native, b - negative control, c - after cryopreservation. Green colour fluorescence (FDA) represents viable cells, red colour fluorescence (EB) represents dead cells.

Moreover, it was found, that DMSO itself polymerizes sodium alginate when 1% of sodium alginate is added to 15 - 50% DMSO solutions in 0.9% NaCl (v/v). At the same time, polymerization does not occur in the presence EDTA. It was observed, that EDTA dissolves the alginate spheres, which were formed by polymerization with DMSO.

Staining with FDA and EB revealed significant lowering of viability of the cells in alginate microspheres after cryopreservation in comparison to the non-cryopreserved control, which was at the same time significantly higher in comparison to the negative control (Figure 6, a-c). This findings correspond well to the data of other researchers [2].

Analysis of metabolic activity of alginate microspheres with MTT test was technically inconvenient due to the challenges in formazan extraction from the alginate structures. Resazurin reduction test showed statistically significant decrease of metabolic activity after cryopreservation (Table 1).

Phenotypic characterization of cells isolated from tissues before and after cryopreservation

In this study we were able to isolate viable cells from all studied placental tissues and multicellular bioengineered constructs after cryopreservation. The

number of cells isolated after cryopreservation of the studied tissues was approximately 40-50% lower in comparison to the number of cells isolated from these samples before cryopreservation (data not shown). It is important to note that there is a great variation in amounts of cells that can be isolated from the native tissues of individual donors. Therefore, obtaining of statistically significant data was not feasible and the stated percentage differences are based on direct experimental observations. However, these findings may bring important information for development of biobanking technologies for such samples.

Cells isolated from the studied tissues before and after cryopreservation were analyzed for the surface markers typical for MSCs (Figure 7). We have previously published phenotypic analysis of the cells derived from placental tissues [23, 24, 42]; however, it was important to complete and systemize such data in the frames of this study.

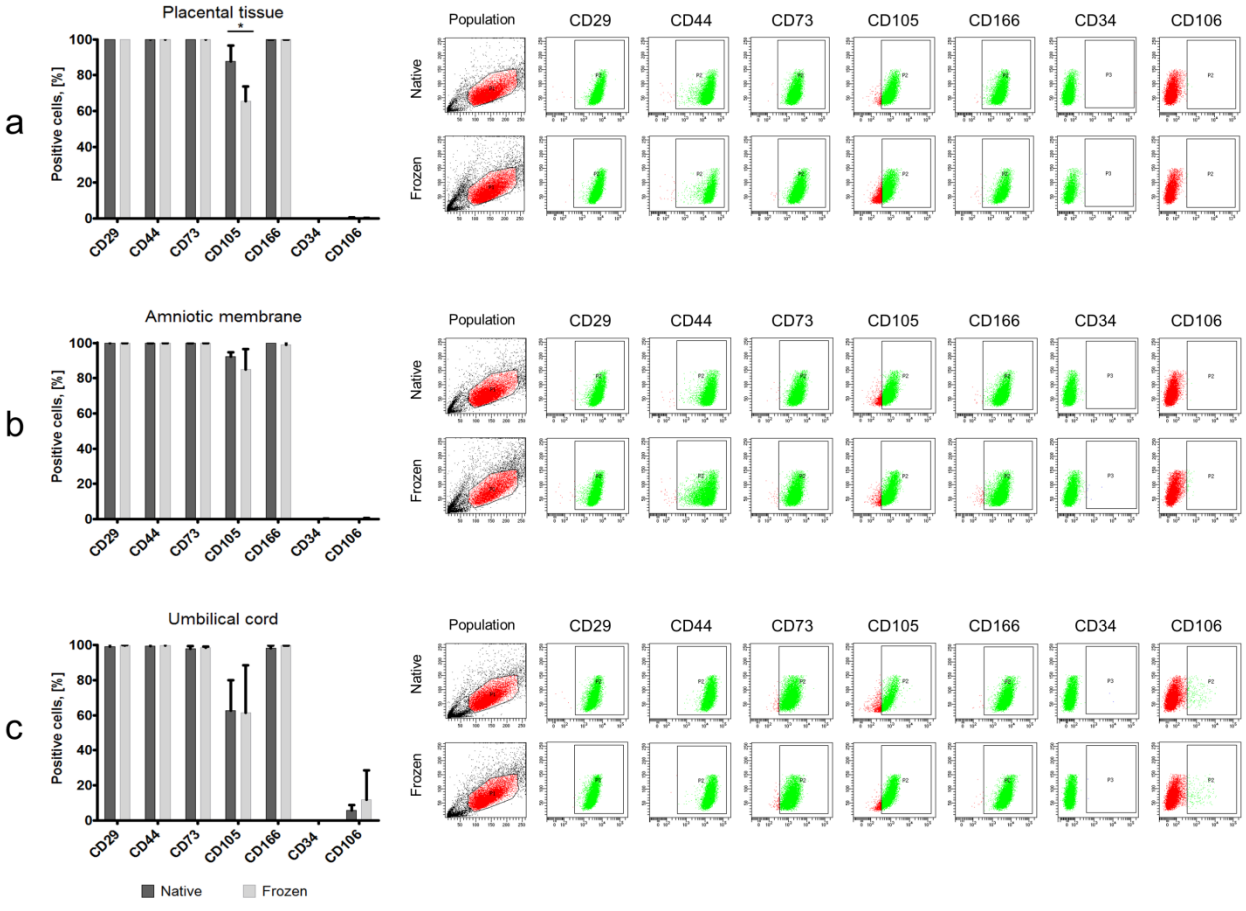

Figure 7. **Phenotypic characterization of cells isolated from the studied tissues before and after cryopreservation.** Flow cytometry analysis for the specific MSC cell surface markers. Typical Dot Plots and percentage of cells positive for CD29, CD44, CD73, CD105, CD166, CD44 and negative for CD34 and CD106: a – cells isolated from placental tissue; b – cells isolated from amniotic tissue; c - cells isolated from umbilical cord. *- $p<0.05$.

Flow cytometry analysis demonstrated that expression of CD29, CD44, CD73, and CD166 surface molecules was over 99% of FSC and SSC gated populations in all of the studied cell samples independently of their origin (placenta, amnion, or umbilical cord) and independently of the tissue condition (native or after cryopreservation). At the same time, all studied cells were negative for CD 34 marker. This demonstrates the highly preserved homogeneity of isolated MSC populations with expression level parameters considered as conventional for this cell type and also confirmed by the other authors [45, 46]. Interestingly, difference in percentage of CD105 positive cells in placenta-derived cells was observed. In cell cultures derived from the native tissue, the number of CD105 positive cells was 87.6 ± 8.9%, while cells derived from the frozen tissue were 65.6 ± 8.2% positive for this marker. The percentage of CD105 cells in the cell cultures extracted from amniotic membranes and umbilical cord did not differs significantly between the samples derived from native and frozen tissues, being 92.3 ± 2.4% (native) and 85 ± 11.4% (frozen) in the amnion-derived and 59.2 ± 20.9% (native) and 61.4 ± 27% (frozen) in the umbilical cord-derived cells. Number of CD106 positive cells in placenta and amnion derived cell cultures was close to 0% both in the cells derived from the native and frozen tissues. However, 5.6 ± 3.1% of CD106 positive cells were detected in the population derived from the native umbilical cord tissue as well as 11.7 ± 16.9% in the cells derived from the frozen umbilical cord tissue.

Studied bioengineered constructs (spheroids and alginate microspheres) contained the cells isolated from the native placental tissue. Profile of expression of the studied surface markers was statistically similar to the profile of placental cells isolated from the native tissue (Figure 6) and did not differ significantly between the cells isolated from native and cryopreserved constructs of both types (data not shown).

Pluripotency genes expression in cells obtained from native and frozen tissues

It is known that placental derived cells possess residual expression of pluripotency genes [47, 48]. Therefore, the cells isolated from the studied placental tissues before and after cryopreservation were analyzed for expression of Oct4, Sox2, Nanog, Klf4, Lin28, and c-Myc genes. It was found, that cryopreservation procedures did not affect significantly the levels of expression of the studied genes (Figure 8).

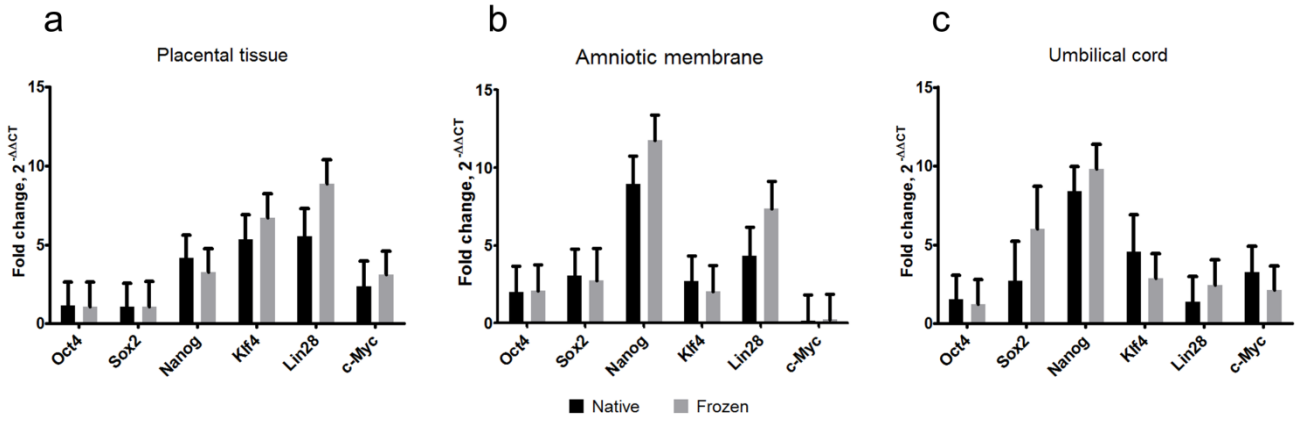

Figure 8. **Expression of pluripotency genes by cells isolated from the studied tissues before and after cryopreservation.** qPCR analysis for expression of pluripotency genes Oct4, Sox2, Nanog, Klf4, Lin28, and c-Myc: a – cells isolated from placental tissue; b – cells isolated from amniotic tissue; c - cells isolated from umbilical cord. Results shown as relative expression against bone marrow MSCs of the same passage.

Discussion

The possibility to save millions of human lives annually, along with the opportunity to significantly improve the life quality, would be realistic with availability of cells, tissues, organs, or clinical-grade bioengineered constructs for transplantation [1, 26, 49]. Ready-to-use accessibility to such biomaterial would exceedingly promote development of regenerative medicine, tissue engineering, and drug discovery. However, the reality of these perspectives is highly challenged by limitations in the shelf life of organs, tissues, and bioengineered constructs, which leads to insufficiency in availability, storage, transportation, certain aspects of necessary quality and safety control, as well as other parameters in supply chain [1, 39, 50].

Cryopreservation techniques with application of subzero temperatures is among the only feasible opportunities to overcome such challenges [1, 36, 39, 50]. At the same time, successful outcome of cryopreservation procedures is related to efficient implementation of numerous factors and parameters intended to minimize cryodamage, such as components of cryoprotective media, cooling and thawing rates, characteristics of ice crystal formation within the samples, and other [51, 52].

Placenta as a rich source of biological material with unique properties, determined by developmental peculiarities of this temporary organ, attracts significant interest of clinicians and researchers from the field of regenerative medicine, tissue engineering, and biobanking [4, 5, 7, 26]. Understanding mechanisms of cryodamage of natural and bioengineered multicellular structures of placental origin would allow developing efficient approaches for improvement of cryopreservation techniques with perspectives to clinical application as well as to extrapolation of these findings on the other tissues and tissue engineered constructs.

In this study, cryopreservation of placental cells, tissues, and multicellular bioengineered constructs revealed a range of consistent patterns that allow concluding on the mechanisms of cryodamage.

Cryopreservation of placental villi was accompanied by minimal crystal formation inside the villi during the temperature drop. The crystals grew during thawing, damaging the mesenchyme. The size of the crystals corresponded to the size of ruptures and extension of the mesenchyme, which was revealed during histological studies. The crystals formed inside the tissue were smaller in comparison to the crystals of extracellular environment. At the same time, majority of the cells remained viable after cryopreservation according to the confocal microscopy analysis of EB/FDA staining, as well as MTT and resazurin reduction tests. Importance of the ice crystal formation during freezing and thawing processes is among the key factors for ensuring successful cryopreservation outcome [52, 53]. Therefore, reduction of recrystallization processes during thawing should be targeted in order to improve the integrity and viability of placental villi after cryopreservation. At the same time, cell density and intercellular fluid of placental villi are optimal for cryopreservation among the studied samples. Generation of bioengineered constructs with similar features may be perspective for achieving high viability outcome after cryopreservation. Huppertz et al. also showed the possibility to cryopreserve placental villi explants with application of 3 M DMSO with retention of morphological parameters, lactate dehydrogenase release, and placenta protein 13 secretion on the levels similar to the non-cryopreserved samples [25].

Amniotic membrane is not only undergoing intensive research, but also successfully applied since decades in ophthalmology and surgical practice with pronounced wound healing properties, determined by the presence of biologically active cells in the epithelial and stromal layers that deliver growth factors and cytokines with anti-fibrotic, anti-inflammatory, anti-bacterial, and anti-immunogenic action [7]. Perepelkin et al. were able to cryopreserve

amniotic membrane with their own protocol with the yield of viable cells in both the stromal and epithelial layers [54]. In our analysis of cryodamage occuring during *cryopreservation of amniotic membrane*, the ice crystals were mainly forming between the dense connective tissue fibers with lesser amount of cells. Such crystals were more structured, but they did not have significant impact on the layer of cells, which is mainly located at the membrane's surface. The size of these crystals was similar to those in cryopreservation medium, but these crystals were oriented along the tissue fibers. In our opinion, the cells of amniotic epithelium were damaged to a higher extent during the standard preparation procedures rather than cryodamage itself. Condition of the tissue after cryopreservation may be adequately analyzed with vital staining and metabolic tests. These tests evaluate condition of the cells located on the membrane's surface. Laurent et al. also suggest various approaches for assessment of cryopreservation efficiency for the amniotic membrane with intended application in biobanking [55].

Great attention of researchers and clinicians is traditionally attracted by umbilical cord blood cells and umbilical cord serum [7, 56]. At the same time, umbilical cord tissue itself is seen as a source of MSCs for potential application in regenerative medicine [38, 57]. Moreover, Yang et al. were able to isolate viable MSCs after cryopreservation of umbilical cord tissue explants in the medium containing 10% DMSO with efficient outcome even after repeated freeze/thaw/explant culture cycles [38]. We have also achieved high rates of viability after cryopreservation of *umbilical cord tissue* in our study. However, assessment of the outcome of cryopreservation of umbilical cord was accompanied with certain technical challenges. This was firstly related to methodological difficulties in making thin enough sections from the highly elastic umbilical cord tissue without mechanical cell injury (especially for cryomicroscopy); secondly, to the poor permeability of certain dyes through the connective tissue; thirdly, to the low number of cells in the umbilical cord; and, fourthly, to the relatively low metabolic activity of this tissue. The most suitable

among the applied methods of evaluation were the staining with EB, which penetrates this tissue well, and resazurin reduction test, which lasts 24 hours and is sensitive to the tissues with low metabolism. According to these methods cryopreservation of umbilical cord tissue allows receiving significant amount of viable cells after thawing.

Along with natural tissues, artificial bioengineered multicellular constructs, such as spheroids or alginate microspheres, are intensively developed for a wide range of biomedical surveys, with the eventual goal of application in regenerative medicine [57, 58]. However, we observed that viability outcome after *cryopreservation of spheroids*, where the cells are connected by intercellular bonds, is rather low. The most pronounced damage was observed in recrystallization phase during warming of the sample. Two main reasons play key role in the spheroid destruction: first – recrystallization processes inside and on the surface of spheroids; second – weakening of the adhesive properties of cells due to the action of DMSO. These changes were observed with both confocal and light microscopy. Spheroids became disintegrated, releasing individual cells. In addition, there was a degradation of individual cells, especially those that were located on the surface of spheroid. At the same time, metabolic characteristics in the culture of spheroids after cryopreservation did not differ significantly to the cell culture in monolayer, which we have observed in our previous study [24].

In this work, our previous analysis of the processes occurring during *cryopreservation of alginate microspheres* with MSCs [2, 3, 29] is supplemented with the novel data on cryomicroscopic analysis of the mechanisms of cryoinjury. This data indicates on heterogeneity of ice in the alginate microspheres during cryopreservation. The major amount of the volume of observed samples was filled with the larger sized crystals, while portion of the smaller sized crystals was minor. Smaller sized crystals began to melt at the lower temperature range during thawing in comparison to the larger crystals. We hypothesize the influence of interaction of alginate with DMSO

and redistribution of DMSO in the samples during cryopreservation process. This hypothesis was confirmed with the fact that heterogeneity in the ice factions was not observed in the case of cryopreservation of alginate without DMSO. Additionally, we determined experimentally that DMSO solutions polymerize alginate at concentrations above 15%. At the same time, the fact that alginate microspheres, obtained with such polymerization with DMSO, became dissolved in the EDTA solution and formation of microspheres in the presence of EDTA was not possible indicates that DMSO binds to the alginate in a non-covalent manner, similar to chelation of calcium ions. Cryomicroscopic analysis showed that majority of the damaged cells was located between the ice crystals and on their boundaries. During thawing the cavities filled with cellular contents were observed. Previous studies also suggest that survival of cryopreserved cells cultured in microspheres is lower in comparison to the cells cryopreserved in suspension [2]. While measurement of metabolic activity of cells directly in the microspheres was challenging due to the properties of alginate, in our previous work we demonstrated metabolic activity of the cells after isolation from alginate microspheres before and after cryopreservation, also in comparison to the cells cryopreserved in suspension in a non-human primate model [3].

In addition to analysis of biophysical parameters of the influence of low temperatures on the studied samples it is very important to perform phenotypic evaluation before and after cryopreservation. The pattern of molecules expressed on the cell surface is not only essential for their characterization, but also for their proper functioning. In our previous studies, we have analyzed cells isolated from placental tissues as primary cultures as well as in long-term cellular culturing, including the studies before and after cryopreservation of the cells [23, 24, 42]. Here, we observed stability of expression of MSC and pluripotency markers by placental MSCs after low temperature storage and in long-term cultures. Genomic stability of MSCs derived from cryopreserved placental tissues in the long-term culture was also confirmed by other

researchers with karyotyping, array-comparative genomic hybridization and microsatellite genotyping methods [30]. In contrary to the previous studies, in this work we characterize the cells isolated from cryopreserved tissues and bioengineered constructs rather than cryopreserved cellular suspensions which were isolated from the native samples.

Conclusions

Cryopreservation of placental villi with 10% DMSO and cooling rate of 1K/min allows obtaining high survival efficiency of cells and preservation of the tissue structure after thawing. In our opinion such outcome could be explained by the moderate thickness and sponginess of this tissue, allowing good permeability for DMSO and resulting in formation of the ice crystals smaller in size in comparison to cryopreservation medium. The cell damage mainly occurs during thawing due to recrystallization. The survival outcome could probably be improved by reducing the recrystallization and acceleration of the thawing rate. Cryopreservation of amniotic membrane and umbilical cord enables survival of significant number of cells. Moreover, viable cells in sufficient amounts could be isolated from all of the studied placental tissues after cryopreservation.

Cryopreservation of spheroids is connected with certain challenges. Firstly, spheroids are destroyed due to the loss of adhesive properties under the impact of DMSO and, secondly, due to the internal crystal formation, which makes them less stable during cryopreservation. The main mechanism of cryodamage to the cells in the alginate microspheres is associated with the damage on the borders of large crystals. At the same time, due to the interaction of alginate with DMSO, the crystal formation is not homogenous and less predictable.

Importantly, cryopreservation procedures did not alter the expression of MSC markers and pluripotency levels in the cells isolated from all studied tissues, except for the minor alteration in CD105 expression in placental villi.

Preservation of natural and bioengineered placental material after thawing can be efficiently evaluated with EB/FDA staining, as well as with MTT and resazurin reduction tests.

Altogether, these results allow concluding that various tissues of placental origin can be cryopreserved with sufficient viability outcome. Generation of novel bioengineered constructs suitable for freezing prospectively require a concept of a spongy matrix, similar to embryonic extracellular environment, which, for example, could be based on collagen and/or elastin fibers with glycosaminoglycans.

Data Availability

All relevant data is available in the manuscript.

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